THE STEROLS OF DEHYDRATED ALFALFA AND CEREAL GRASSES

by

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INTRODUCTION

Sterols can be converted by chemical methods to synthetic sex hormones and compounds having anti-rachitic properties. At present the sterols for these purposes are obtained from soybean seeds because of the quantity present and the ease with which they can be isolated. It is possible that the sterols of alfalfa also may be suitable for such uses. The sterols are thus of interest in an investigation of the industrial utilization of dehydrated alfalfa which is being conducted by the Department of Chemistry. Because of the potential use of cereal grasses for dehydration, also, the sterols of some of the latter were included in this study.

REVIEW OF LITERATURE

A number of methods have been published for the quantitative determination of cholesterol in blood and animal tissue and of sterols in vegetable oils. A gravimetric procedure involves the use of digitonin as a precipitating agent for the sterols. A colorimetric procedure which is widely used is based on the Liebermann-Burchard reaction, in which a blue or green color is produced when acetic anhydride and sulfuric acid are added to the sterol solution. The former is based on the work of Windaus (1). Bloor (2) used the digitonin method for determining the amount of cholesterol in blood. Schoenheimer and Sperry (3) precipitated cholesterol as the digitonide and carried out the Liebermann-

Burchard reaction on this complex. Sperry (4) showed the colorimetric procedure to be as accurate as the gravimetric method.

Little work has been done on the plant sterols. The many pigments present offer complications. Wall and Kelley (5) made use of the chromatographic separation of chlorophylls and xanthophylls from the carotene and sterols. Zechmeister (6) removed carotene by precipitating it with iodine. Wall and Kelley (7) have modified many of the foregoing procedures and have adapted them for the determination of the sterols in dehydrated plant meals.

Most of the phytosterols that have been characterized were isolated from seed oils. Little has been done with the sterols of the vegetative parts of plants, however. This perhaps is due to the lower sterol content and the greater number of interfering substances which are present in the leaves and stems. Heyl, Wise and Speer (8) isolated spinasterol from spinach. Heyl and Larsen (9) characterized γ-spinasterol from a glucoside in spinach. Fernholz and Moore (10) isolated α-spinasterol from alfalfa, and Fernholz and Ruigh (11) have suggested a structure for it.

EXPERIMENTAL

Determination of Sterols in Dehydrated Plant Meals

The method of Wall and Kelley (7) was used, which was essentially as follows: The dried plant material was extracted with Skellysolve B in a Soxhlet extractor. An aliquot of this extract was adsorbed on a magnesium oxide column. The sterols and carotene

were eluted by a mixture of acetone and Skellysolve B. Iodine was added to the eluate and the carotene was precipitated as the insoluble iodide. This precipitate was filtered off, and the excess iodine in the filtrate was removed with sodium thiosulfate. The solution was evaporated and the residue dissolved in hot absolute ethanol. Digitonin was added to precipitate the sterol. The sterol digitonide was filtered off and weighed. In calculating the quantity of sterol from the weight of the digitonide, the gravimetric factor 0.253 was used. One mole each of sterol and digitonin is involved in the complex (7).

The sterol digitonides obtained by the gravimetric procedure were used to establish standard curves for use with the colorimetric procedure. The sterol digitonide was dissolved in acetic acid, and aliquots of the solution were allowed to react with acetic anhydride and concentrated sulfuric acid. A Beckman spectrophotometer was used to measure the intensity of the color produced. These values were plotted to obtain the standard curves. Wall and Kelley (7) show the sterol digitonide of alfalfa to have an absorption maximum at 680 mu. The absorption maxima for the sterol digitonides of brome grass and the wheat plant (Fig. 1) were found to be 680 mu. also.

When subjected to the Liebermann-Burchard reaction, the sterol digitonides of alfalfa were reported by Wall and Kelley to have a time-density curve characteristic of spinasterol; i. e., a maximum color density was produced immediately upon addition of the reagent. The density decreased with time, and approached constancy at 30 minutes. $E^{1\%}_{1cm}$ values for the sterol digitonide obtained in

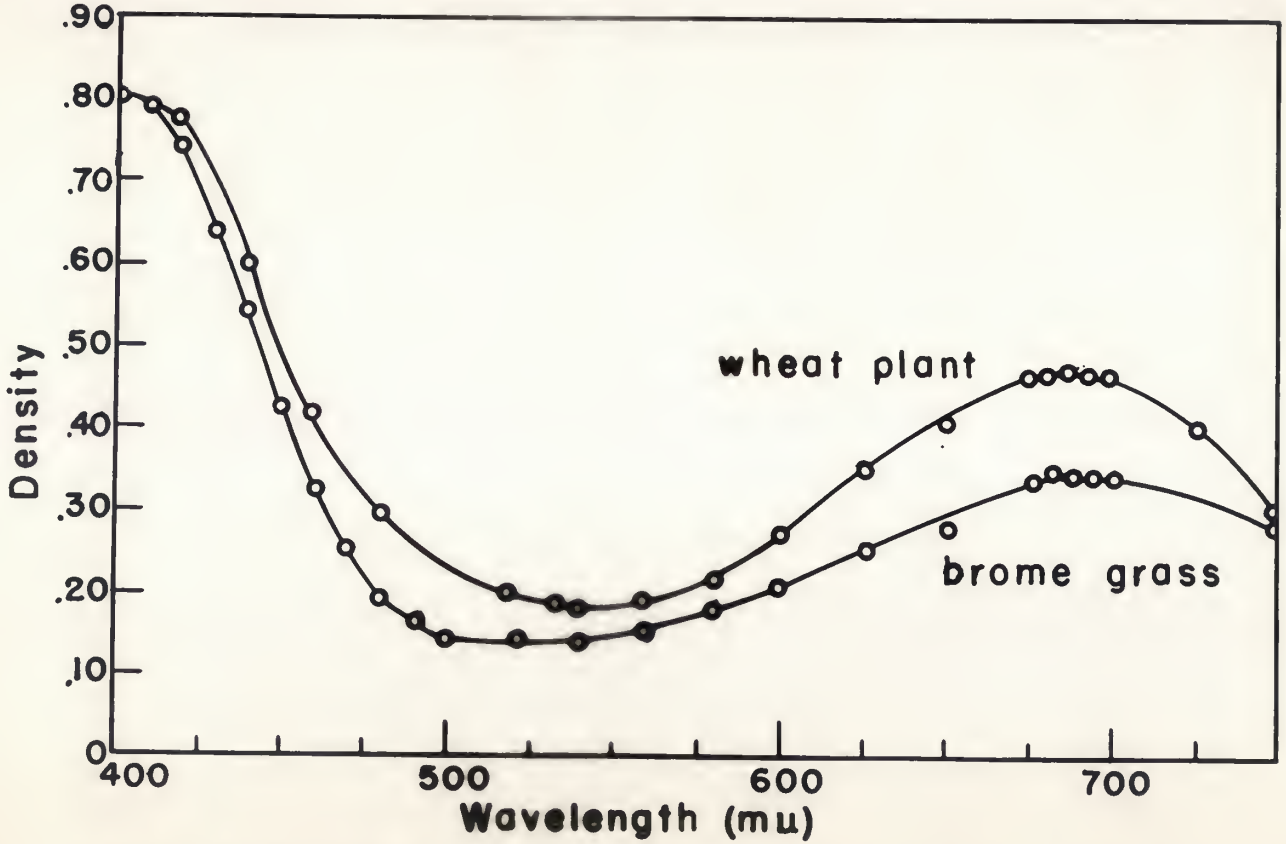

Fig.1. Absorption curves of the digitonides of the sterols in brome grass and wheat plant.

this work were calculated to be 77, as compared with Wall and Kelley's value of 79.

The time-density curve for the sterol digitonide of brome grass when read at 680 mu. is markedly different from that of the sterol digitonide of alfalfa (Fig. 2). The color density is at a minimum in the early stage of the reaction and is at a maximum at about 30 minutes. This type of reaction is characteristic of the sitosterols (7). Because of these differences in color development, it was necessary to prepare standard curves for each plant to be analyzed.

The $E^{1\%}_{1cm}$ value for brome grass sterol digitonide was found to be much lower than the reported value for sitosterol. The possibility that this might be due to the presence of saturated sterols was investigated, since saturated sterols are precipitated by digitonin but do not undergo the Liebermann-Burchard reaction. The method of Anderson and Nabenhauer (12) was used. The sterols in a chloroform solution were shaken with acetic anhydride and concentrated sulfuric acid. The unsaturated sterols passed into the acid layer and formed a deep blue complex. The saturated sterols remained in the chloroform and were determined gravimetrically. A small amount of saturated sterol was found, and with this correction an $E^{1\%}_{1cm}$ value of 30 was obtained. The $E^{1\%}_{1cm}$ value of β-sitosterol is 29.2 (7).

The time-density curve obtained with the digitonide of the wheat plant sterols was the same as the curve of the brome grass sterol digitonide (Fig. 2). It, too, is a sitosterol. The $E^{1\%}_{1cm}$ value for the wheat plant sterol digitonide was 26.6. When the

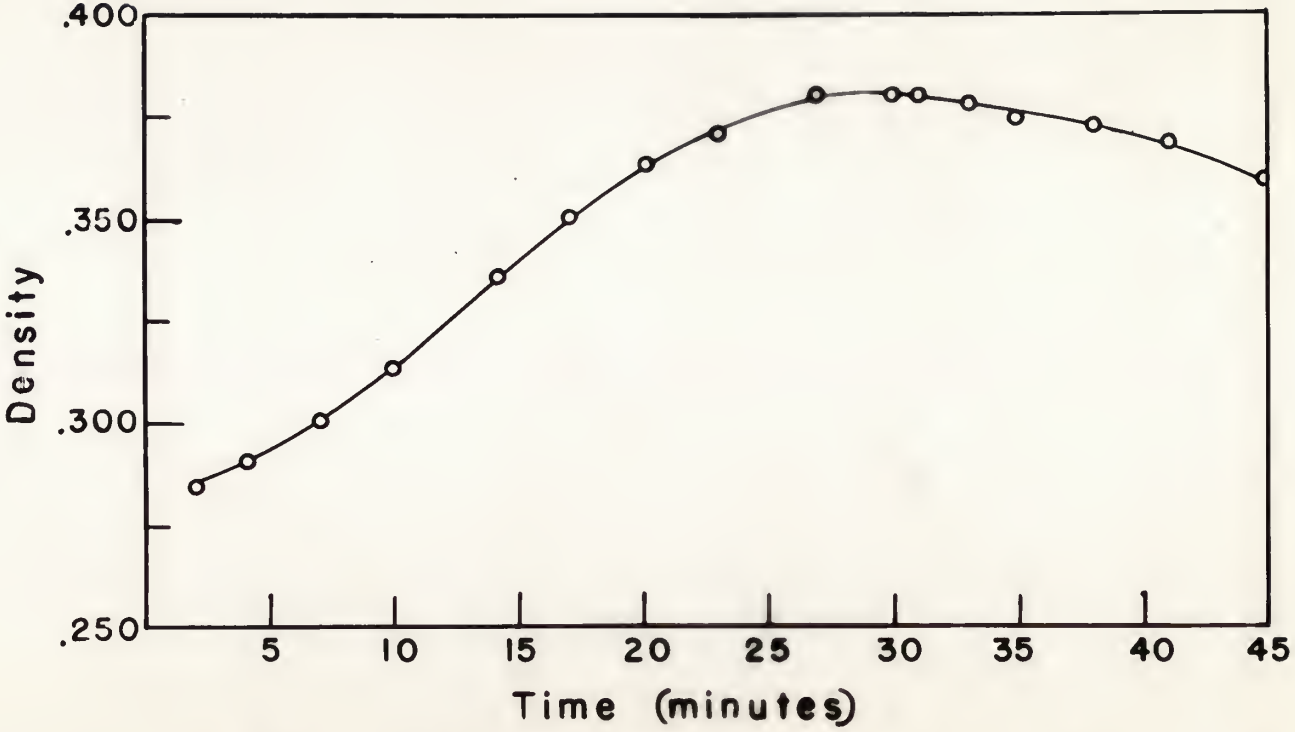

Fig.2. Time-density curve for the sterol digitonide of brome grass.

wheat plant was examined for saturated sterols, only a trace was found.

Effect of Stage of Growth and Nitrogen Fertilization on Sterol Content

The colorimetric procedure was used in determining the effect of maturity on the sterol content of alfalfa, brome grass, and the wheat plant, and the effect of nitrogen fertilization on the sterol content of brome grass. An aliquot of the Skellysolve B extract was chromatographed to remove chlorophylls and xanthophylls. Carotene was precipitated with iodine. A portion of the purified extract was placed in a conical centrifuge tube and the solution evaporated to dryness. The residue was washed with Skellysolve B and centrifuged. The Skellysolve B was decanted, and the washing repeated with another portion of Skellysolve B. The precipitate was taken up in acetic acid. Acetic anhydride and concentrated sulfuric acid were added. The density was measured at 680 mu. 30 minutes after the addition of the sulfuric acid.

Figure 3 shows the effect of maturity on the sterol content of alfalfa. The last sample was taken when the plants were in early bloom. It will be seen that there was a small decrease in the sterol content as the plants became older.

Sterols may exist in a combined condition as esters, and when combined are not determined by the methods described above. Alfalfa does not contain combined sterols (7). Nothing was found, however, concerning the condition of the sterols in brome grass and the wheat plant. To investigate this, an aliquot of the plant ex-

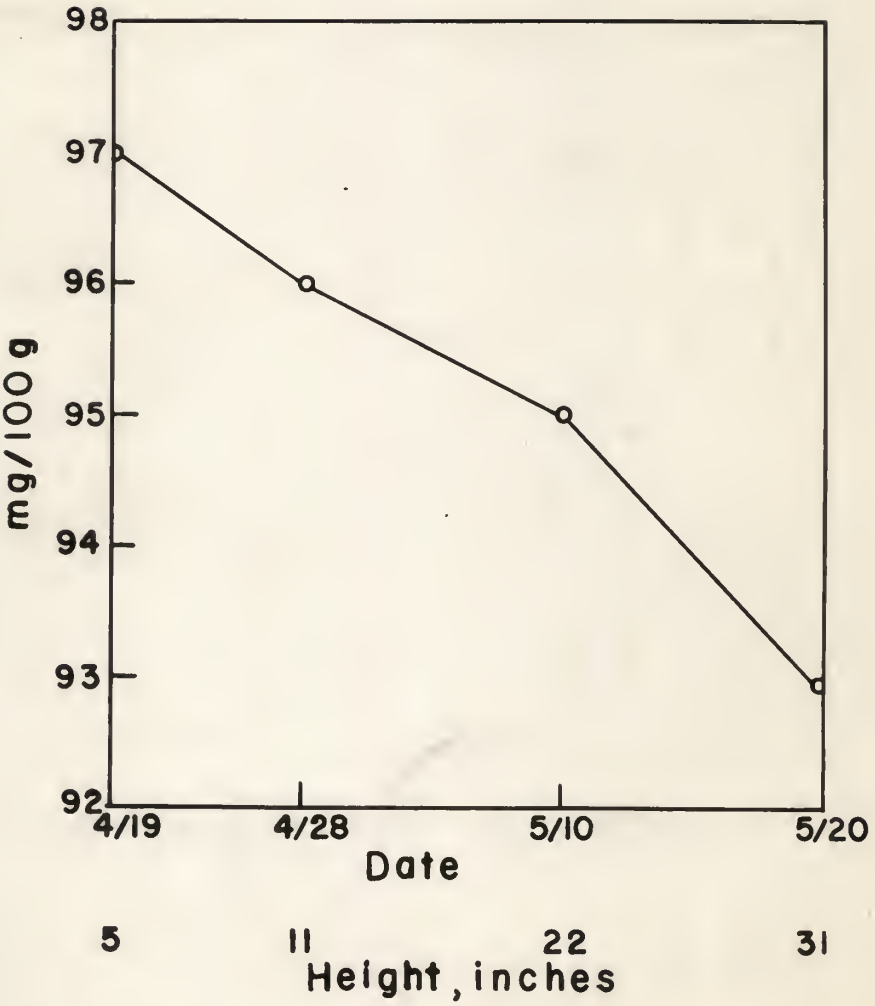

Fig.3. Effect of stage of growth on the sterol content of alfalfa.

tract was saponified and the unsaponifiable fraction was extracted with Skellysolve B. Xanthophylls were removed by shaking with 90 per cent methanol, and sterols were determined colorimetrically as described above. The result thus obtained represents total sterols. A large proportion of the sterols of brome grass was found to be combined. In the wheat plant, combined sterols were found only in the final sample, when the plant had started to head.

Figure 4 shows the effect of stage of growth on the sterol content of brome grass which had been fertilized with 100 pounds of nitrogen per acre, the nitrogen being applied as ammonium nitrate. As the plants became older, total sterols increased slightly while free sterols decreased. The resulting increase in combined sterols is in accord with the tendency of plants to condense smaller molecules into larger ones as they approach maturity.

The effect of nitrogen fertilization on the sterols of brome gasss in presented in Fig. 5. The latter data were obtained from plots which had been fertilized with ammonium nitrate and which were sampled at the time the plots were cut for hay after vegetative growth had ceased. From Fig. 5 it will be seen that both free and total sterols increased as the nitrogen application was increased from zero to 200 pounds.

Figure 6 shows the effect of stage of growth on the sterol content of the wheat plant. The final sample was obtained at the early heading stage. No combined sterol was detected except in the last sample obtained, when the combined form represented about

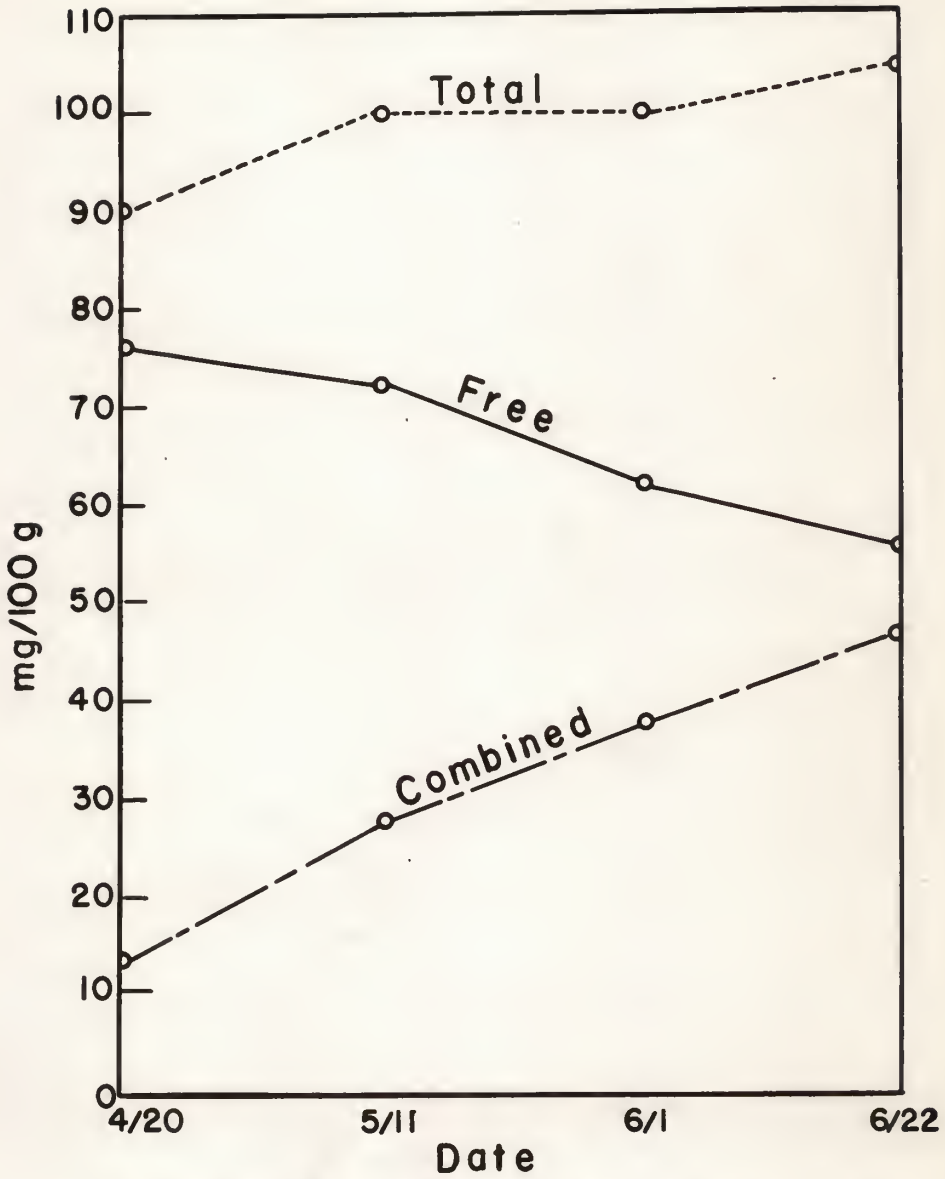

Fig. 4. Effect of stage of growth on the sterol content of brome grass.

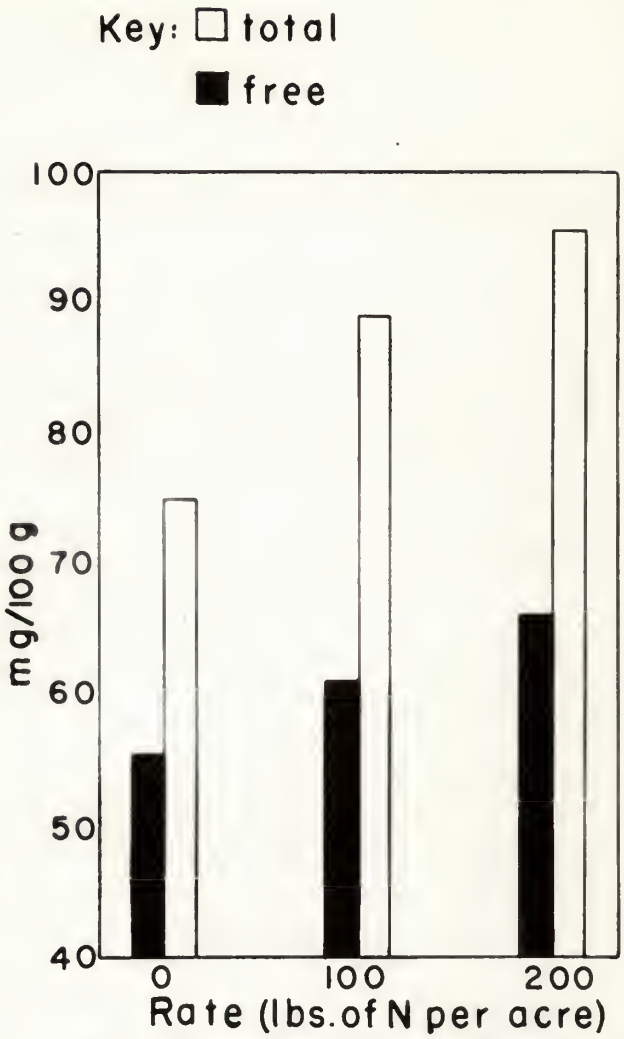

Fig. 5. Effect of fertilization with ammonium nitrate on the sterol content of brome grass.

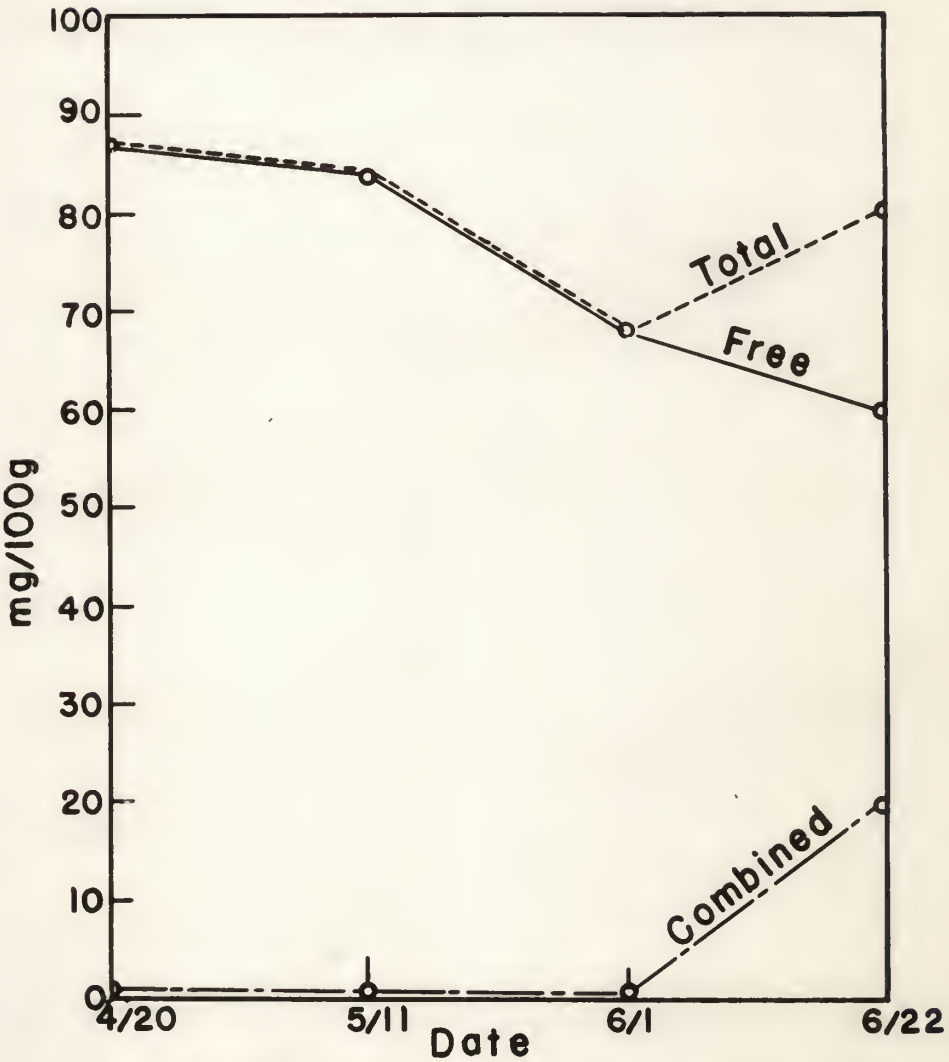

Fig.6. Effect of stage of growth on the sterol content of the wheat plant.

25 per cent of the total sterols. Free sterols decreased in a regular manner throughout the growth period.

Isolation of a Sterol from Brome Grass

The time-density curve for the sterol digitonide of brome grass (Fig. 2) indicated it to be a sitosterol. The isolation of this sterol was attempted; a modification of the method of Fernholz and Moore (10) was used. Six kilograms of dried brome grass were exhaustively extracted with Skellysolve B. Chlorophyll and xanthophylls were removed by adsorption. The residue obtained weighed 57 grams. The residue was saponified and the unsaponifiable fraction was extracted with Skellysolve B. The Skellysolve B solution was evaporated to dryness and the residue was dissolved in hot acetone. On cooling, the waxes precipitated and were removed by filtering. The filtrate was evaporated to dryness. This residue, which weighed 20 grams, was dissolved in Skellysolve B and was washed 10 times with 100 ml of 95 per cent methyl alcohol. The methanol washings were combined and evaporated to dryness. A residue weighing 7 grams was obtained. This residue was dissolved in hot methyl alcohol, from which the sterols crystallized on standing. A yield of 100 mg. was obtained. The sterol was recrystallized three times from methyl alcohol. It had a melting point of 129-130° C., which did not change on further recrystallization. The acetate was made by refluxing the sterol with acetyl chloride for 15 minutes. It had a melting point of 169-170° C. which did not change on recrystallization. This ester was saponified, and the sterol obtained after recrystallization from methyl

alcohol had a melting point of 128-129° C.

Separation of Sterols from Carotene Concentrates

The carotene concentrate which can be prepared from dehydrated alfalfa meal for use as a vitamin supplement for feeds also contains part of the sterols which were present in the meal. For example, in one experiment 1000 grams of dehydrated alfalfa meal yielded 5.2 grams of a carotene concentrate. The concentrate contained 1.5 per cent sterol. This compares with 0.2 per cent sterol in soybean oil, the present commercial source (11). To utilize the sterols of alfalfa and cereal grasses industrially, and still retain the carotene for feeding purposes, a method must be found for separating them from the carotene without destroying the biological activity of the latter.

A chromatographic separation of the sterols from the carotene was attempted with the following adsorbents: $Ca_3(PO_4)_2$, $Ca(OH)_2$, Na_2CO_3, $MgCO_3$, $CaHOP_4$, alumina, norite, and sorghum starch. None of these adsorbents effected a separation of the sterols and the carotene.

Attempts to separate the sterols from the carotene by chemical methods were investigated. Yoder (13) reacted sterols with oxalic acid in an anhydrous solvent, producing a crystalline sterol-oxalic acid addition product. Sobel and Spoerri (14, 15) reacted some of the sterols with pyridine sulfur trioxide and obtained crystalline pyridonium sterol sulfates.

Neither the method of Yoder nor that of Sobel and Soerri accomplished the separation of the sterols from the carotene in the

concentrates which were used in this work. These methods, however, were developed and used with materials which contained less complicating substances than were present in the carotene concentrates from dehydrated alfalfa. Yoder used his method on wool fats and seed oils, while Sobel and Spoerri worked with yeast and animal tissue. Thus, they were not troubled by the high concentrations of carotene, phosphatides, and waxes which are encountered in alfalfa extracts. It is probable that such materials also were responsible for the lack of resolution by means of adsorbents, since even neutral fat is known to decrease the adsorbability of carotene on a magnesia column. Perhaps one or more of the adsorbents used would effect a separation if the pure sterol and pure carotene were mixed and chromatographed.

DISCUSSION

At least four plant sterols have been characterized sufficiently so that they are considered to be individual substances. A number of others which have been described may be individual sterols or a mixture of the sterols which have been characterized (16, p. 801). The melting point of the sterol isolated from brome grass does not agree with any of the melting points of the well characterized sterols, nor does the melting point of its acetate agree with any other sterol acetate found in the literature. There is insufficient evidence to conclude that this sterol is or is not a single entity. One might suspect that it is actually a mixture, since the sterols of other plant materials have been found to be multiple in nature (17, p. 386).

The data indicate that lipid concentrates prepared from dehydrated alfalfa meal contain about seven times as much sterol as soybean oil, which is the present source of stigmasterol for hormone production. Because of the greater difficulty of isolating the alfalfa sterol, it probably will be unable to compete successfully with soybean sterol unless the carotene can be recovered for use as a vitamin supplement, and thus bear part of the expense of production. Incidental to the problem under investigation was the isolation of other substances, apparently phosphatides and waxes, from the lipid concentrates. These also may have industrial significance. Further study is needed to determine the desirability of fractionating crude carotene concentrates for the production of sterols.

SUMMARY

1. The effect of stage of growth on the sterol content of alfalfa, brome grass, and the wheat plant was investigated. The free sterol content in these plants decreased as the plants approached maturity. The combined sterols in brome grass increased with maturity. The wheat plant contained no combined sterols until heads had started to form. At this time, 25 per cent of the sterols was combined.

2. Fertilization with ammonium nitrate caused an increase in both the free and combined sterols of brome grass.

3. The sterols in brome grass and the wheat plant were identified as sitosterols by means of the time-density curves of the digitonides obtained during the Liebermann-Burchard reaction. The

sterol isolated from brome grass had a melting point of 129-130° C. The acetate had a melting point of 169-170° C.

4. All attempts to separate the sterols from carotene concentrates without destroying the carotene were unsuccessful.

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